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Brain Research 915 (2001) 248–255

BRAIN RESEARCH INTERACTIVE

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Interactive report

Concurrent release of ATP and substance P within guinea pig trigeminal ganglia in vivo ☆

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Accepted 27 July 2001

Abstract

Neurons within sensory ganglia have been proposed to communicate via non-synaptic release of a diffusible chemical messenger, but the identity of the chemical mediator(s) remains unknown [*J. Neurosci*. 16 (1996) 4733–4741]. The present study addressed the possibility of co-released ATP and substance P (SP) within sensory ganglia to further advance the hypothesis of non-synaptic communication between sensory neurons. Microdialysis probes inserted into trigeminal ganglia (TRGs) of anesthetized guinea pigs were perfused with artificial cerebrospinal fluid and the collected perfusate analyzed for ATP and SP content using the firefly luciferin–luciferase (L/L) assay and radioimmunoassay, respectively. Significant reversible increases in ATP and SP levels were observed after infusion of 100 mM KCl or 1 mM capsaicin. Ca^{2+}-free ACSF produced an eightfold increase in ATP levels, interpreted as a decrease in activity of Ca^{2+}-dependent ecto-nucleotidases that degrade ATP. In contrast, KCl-induced release of ATP in the presence of normal Ca^{2+} was blocked by Cd^{2+}, a voltage-gated Ca^{2+} channel blocker, illustrating Ca^{2+}-dependence of evoked ATP release. Since ganglionic release of ATP could arise from several neuronal and non-neuronal sources we directly tested acutely dissociated TRG neuron somata for ATP release. Neuron-enriched dissociated TRG cells were plated onto glass tubes and tested for ATP release using the L/L assay. Robust ATP release was evoked with 5 μM capsaicin. These data suggest that ATP is released concurrently with SP from the somata of neurons within sensory ganglia.

Theme: Sensory systems

Topic: Somatic and visceral afferents

Keywords: Neuropeptide; Microdialysis; Pain; Trigeminal ganglia; Substance P; ATP

1. Introduction

The general absence of synaptic contacts and the offstream soma position of neurons in mammalian sensory ganglia have led to the traditional view that each primary afferent neuron acts as an independent sensory information channel that does not affect the activity of neighboring neurons, at least until the first synapse in the central nervous system (CNS) [19]. Recent studies have directly challenged this view by demonstrating that under normal conditions, activity in neighboring neurons elicits functional cross-excitation in the somata of affected sensory neurons [1,2]. The cross-excitation is, in the vast majority of cases, a depolarization that increases the probability of impulse generation in the soma. After nerve injury the probability of such ‘ectopic’ impulse generation is greatly increased; this provides a possible mechanism for hyperalgesia in chronic pain [2,8].

Cross-excitation appears to be mediated by an unidentified chemical messenger(s), released from active neurons and affecting excitability of nearby neurons [1]. It is

☆Published on the World Wide Web on 30 August 2001.

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0006-8993/01/$ – see front matter
PII: S0006-8993(01)02888-8

possible that after inflammation or peripheral nerve injury the cross-excitation is enhanced as a consequence of augmented release of this chemical messenger and/or increased activation of its receptors. For example, a large subset of spinal and trigeminal sensory neurons depolarize in response to application of synthetic substance P (SP) [9,32,33]. Importantly, SP is released from the somata of sensory neurons in vitro [17] and its release within sensory ganglia is greatly increased in vivo after peripheral inflammation [24].

Adenosine-5′-triphosphate (ATP) is also released after stimulation from peripheral sensory nerves [15], and it has been reported that ATP is co-released with other neurotransmitters from autonomic and CNS neurons [18,31,34]. Application of ATP to dorsal root ganglion (DRG) neurons was shown to evoke inward currents via the P2X ion channels [5,37]. The $P2X_3$ purinoceptor subtype is especially concentrated on the somata, peripheral and central terminations of the nociceptive sensory neurons [3,7,10,26]. The $P2X_3$ receptor is transiently upregulated in the trigeminal ganglion (TRG) and DRG after peripheral nerve injury [10,26]. Moreover, purinergic sensitivity develops at the site of chronic nerve constriction injury [6]. These studies suggest that ATP plays a role in nociceptor activation via the $P2X_3$ receptor and raise the possibility that ATP may act as a chemical messenger of cross-excitation within sensory ganglia.

The present study addressed the possibility of ATP release from somata of sensory neurons within sensory ganglia in order to advance the hypothesis of chemically mediated non-synaptic communication between sensory neurons.

2. Materials and methods

2.1. Surgery and microdialysis

Surgery and microdialysis were performed as described previously [24]. Male Hartley guinea pigs (330–510 g) were anesthetized with sodium pentobarbital (37 mg/kg, i.p.), and following tracheal cannulation, placed in a stereotaxic mount. Microdialysis probes (CMA/12, CMA) were inserted into the right and/or left TRGs. Artificial cerebrospinal fluid (ACSF) was pooled above the TRG and mineral oil was used to fill the cranial cavity to minimize ACSF evaporation. ACSF composition was (in mM): NaCl, 125; KCl, 2.5; Na_2HPO_4, 5; NaH_2PO_4, 0.9; $CaCl_2$, 1.2; $MgCl_2$, 1.0; D-glucose, 2.5; 0.025% bovine serum albumin. Body temperature was maintained at 37±0.5°C with a circulating-water heating pad (K-20, Hamilton) and the animal ventilated at 1 stroke per 3 s (Harvard Apparatus respirator, Model 661, tidal volume 2–3 ml). The blood gases were not monitored. The microdialysis probes were continuously perfused (2.5 μl/min) with ACSF using 10-ml gas-tight syringes and an infusion pump (2400-004, Harvard Apparatus).

Capsaicin and $CdCl_2$ (Sigma) were added after appropriate dilution to the ACSF. For high K^+ solutions, NaCl content was reduced to maintain ACSF osmolarity. In some experiments, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 10 mM) was added to a $CaCl_2$-free ACSF, while $MgCl_2$ was appropriately increased to maintain divalent and chloride ion balance. An injection valve was used to switch between control and 'drug' ACSF [20]. Perfusate samples were collected every 20 min into iced polypropylene tubes (0.5 ml; Fisher Scientific), immediately frozen on dry ice and stored (−70°C) until analysis.

2.2. ATP analysis

Release of ATP was determined using the firefly luciferin–luciferase (L/L) assay. L/L reagent (30 μl, CLS II, Boehringer Mannheim or 50 μl, FF2021, Enliten) was added to samples (40 μl) in a cylindrical cuvette, mixed and placed into a luminometer (TD-20/20, Turner Designs). Luminometer settings were delay: 3 s, integration: 20 s, for ATP analysis with the Enliten L/L reagent, and delay: 1 s, integration: 10 s for analysis with the Boehringer Mannheim L/L reagent.

ATP standards (range, 10 pM–10 nM) were measured before each set of microdialysis samples. The recovery of ATP across the dialysate membranes was determined in vitro. Microdialysis probes (perfused with ACSF at 2.5 μl/min) were immersed into a beaker containing ACSF for 60 min, then placed into a beaker containing 1, 10 and 100 nM ATP in ACSF for 100 min each, then back to the ACSF beaker for 40 min. The dialysate (collected every 20 min) and solutions from both beakers were analyzed by the L/L assay. Average ATP recovery by the probes was 11.29±0.95% (n=3 probes). The probes also showed little residual ATP in samples collected after return to control ACSF.

2.3. Radioimmunoassay (RIA)

In some experiments SP content was measured by RIA, after ATP measurements, using methods described previously in detail [24]. Briefly, 96-well Immulon II-coated plates were coated with 100 μl of protein A. Plates were incubated with SP antibody at room temperature for >2 h. Quadruplicate SP standards (0.1–50 fmol/40 μl of experimental ACSF) were loaded onto plates along with the perfusate samples. Samples and standards were incubated overnight at 4°C. Next day, 50 μl of assay buffer with [^{125}I-Tyr^8]SP was added to each well and incubated overnight at 4°C. The wells were washed, blotted dry and radioactivity measured in a gamma counter.

2.4. ATP release from acutely dissociated TRG cells

The methods for dissociation of TRG neurons were similar to those previously described [11,41]. Guinea pigs were deeply anesthetized (60 mg/kg pentobarbital, i.p.), and the TRGs were removed, de-sheathed and cut in small pieces in ice-cold Hank's Balanced Salt Solution (HBSS, Sigma) with 20% fetal bovine serum (FBS, HyClone). TRGs were incubated for 25 min at 37°C in 5 ml of minimal essential medium (MEM, Gibco) with 10% FBS, 0.125% collagenase P (Roche) and 0.02% DNase (Sigma). TRGs were then transferred for 5 min at 37°C to 2 ml of Ca^{2+}- and Mg^{2+}-free HBSS (Sigma) containing 0.25% trypsin (Sigma) and 0.05% DNase. Cells were dissociated by tissue trituration with a series of fire-polished Pasteur pipettes in 5 ml HBSS containing 0.295% $MgSO_4$ and 0.02% DNase.

Dissociated cells were pre-plated for 15 min in 10% MEM–FBS in a 35 mm tissue culture dish (Becton Dickinson). During this period, most of the non-neuronal cells adhered to the plastic surface, and the rest of the cells were re-suspended. 100 μl suspensions were placed in test tubes previously coated with 50 μl of matrigel (1:100 dilution, Becton Dickinson). The supernatant was removed 1 h after plating and 100 μl MEM added. Portions of each suspension tested for ATP were grown overnight on matrigel coated cover slips incubated for 10 min with 10 μg/ml of rhodamine-conjugated lectin glycine Max (SBA, Vector) [12], and the cells counted with phase-contrast microscopy. Fluorescent SBA staining was visualized with a standard rhodamine filter. All round-bodied SBA positive cells were counted as live neurons. All non-round, SBA negative cells were counted as non-neuronal cells. Counts were made from five randomly selected fields on each cover slip.

At 5 h after plating in glass tubes, the neuron-enriched TRG cells were tested for ATP release. L/L reagent (50 μl, CLS II, Boehringer Mannheim) was added to samples in 100 μl MEM and placed in the luminometer. After four consecutive 10 s measurements, capsaicin (in 10 μl MEM, final concentration; 5 μM) was added and the second set of four measurements was performed.

2.5. Statistical analysis

All data are presented as mean±S.E.M. Statistical analyses [one-way and/or two-way repeated measure analysis of variance (ANOVA) with post hoc comparisons, Friedman repeated measures ANOVA on ranks] were used to compare baseline ATP or SP to post-stimulation levels and to ATP levels in samples collected during EGTA or $CdCl_2$ perfusion. Paired *t*-test was used to compare ATP release from TRG cells before and after capsaicin application.

3. Results

3.1. Release of ATP and SP within trigeminal ganglia by local depolarizing stimuli

Following collections of baseline microdialysis samples from the TRG, the control microdialysate solution was switched to one containing 100 mM KCl for 20 min to depolarize TRG neurons. Samples collected during the first hour usually showed high ATP or SP content. Since this could result from injury of surgery and insertion of the microdialysis probes, for the purposes of analysis, we selected the baseline to begin 1 h after commencing perfusion. A reversible significant increase in the amount of ATP was seen after the high KCl infusion (Fig. 1A). The same samples also showed significant and reversible KCl-evoked increases in the amount of SP (Fig. 1B). These experiments showed that both ATP and SP are released in response to neuronal depolarization within the TRG.

3.2. Capsaicin-evoked ATP and SP release

We next measured changes in ATP and SP levels after capsaicin infusion into the TRG. Capsaicin is known to selectively excite polymodal C- and Aδ-nociceptors with subsequent release of SP from their peripheral and central terminations [16]. Infusion of 1 mM capsaicin into the TRG via microdialysis probes significantly increased sampled ATP and SP levels as compared to baseline levels (Fig. 2). These data suggested that ATP is co-released with SP within the TRG following selective activation of polymodal C- and Aδ-nociceptors.

3.3. Calcium dependence of the ATP release

In our previous study, we used a double stimulation paradigm to show that infusion of EGTA with a Ca^{2+}-free ACSF blocks KCl-evoked SP release [24]. Therefore, we used a similar paradigm to determine whether basal and KCl-evoked ATP release within the TRG is also dependent on extracellular calcium levels. Following the first KCl stimulation, the perfusate was switched to Ca^{2+}-free ACSF containing the calcium chelator EGTA (10 mM) for the remainder of the experiment, including the second KCl stimulus (Fig. 3). Surprisingly, after switching to the Ca^{2+}-free, EGTA-containing ACSF, ATP levels in dialysate samples increased to >600% of baseline values, and the subsequent high K^+ stimulus did not induce significant ATP release. The results could not be explained by interference of the EGTA-containing, Ca^{2+}-free ACSF with the ATP assay, because ATP standards dissolved in EGTA-containing, Ca^{2+}-free ACSF were similar to the normal ACSF-containing ATP standards. We reasoned that the large increase in ATP levels during the EGTA-con-

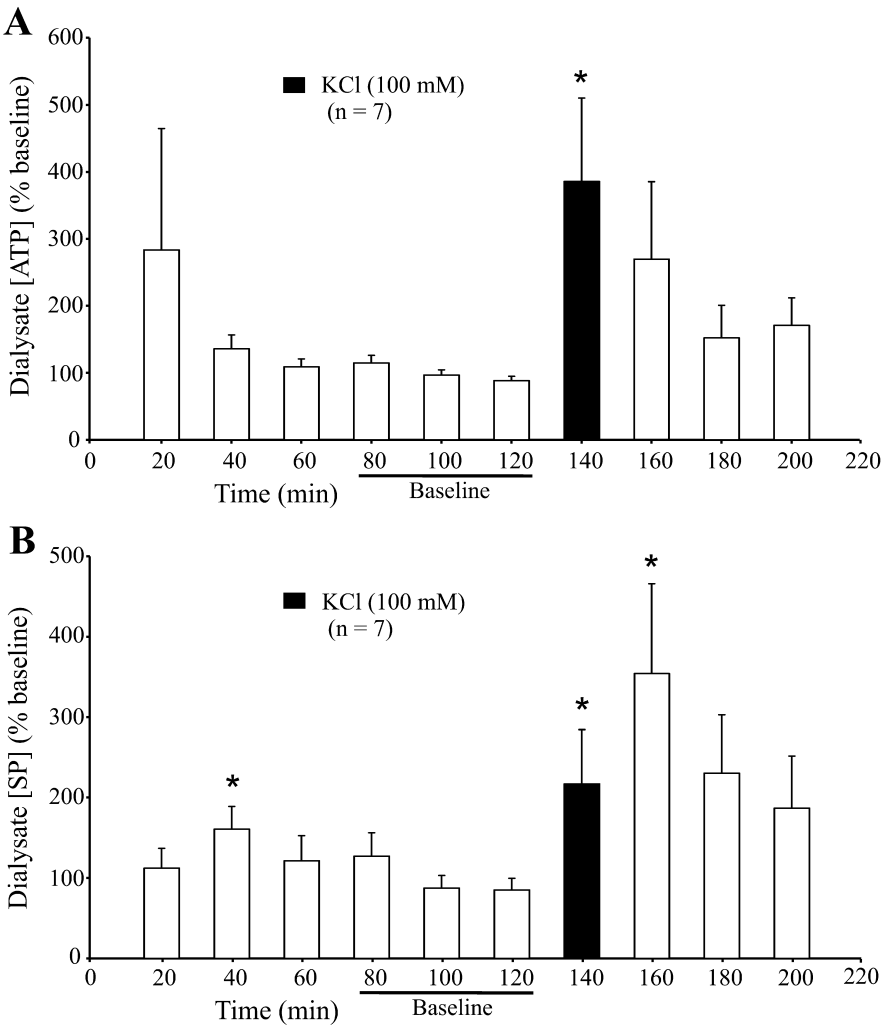

Fig. 1. KCl-evoked release of ATP and substance P within guinea pig trigeminal ganglia in vivo. (A) Summary of seven experiments measuring ATP content in microdialysis samples by the firefly luciferin–luciferase assay. Data is shown as mean % of baseline±S.E.M. before and after infusion of 100 mM KCl. Actual baseline [ATP] was 232.3±106.6 pM. (B) Summary graph of SP levels measured by radioimmunoassay in the same samples after ATP analysis. Actual baseline [SP] was 65.6±31.4 pM. Asterisks denote significant difference ($P<0.05$) from the last sample before KCl-infusion. Baseline for each experiment was measured as the average ATP or SP in the three samples starting 1 h prior to KCl infusion.

taining, Ca^{2+}-free ACSF infusion might be due to interference with the Ca^{2+}-dependent enzymes that degrade extracellular ATP (see Discussion).

In order to block voltage-gated Ca^{2+}-channels while maintaining normal extracellular Ca^{2+} levels, we used Cd^{2+}, which blocks the Ca^{2+} influx through voltage-gated Ca^{2+}-channels [14]. In this experiment, the probes were perfused with normal ACSF followed by 20 min high K^+ stimulus and then they were switched to ACSF with 100 μM Cd^{2+}. Analyses of ATP content revealed that 100 μM Cd^{2+}-containing ACSF did not increase basal release of ATP and prevented the second KCl-evoked ATP release (Fig. 4), suggesting that KCl-evoked release of ATP is dependent on Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

3.4. ATP release from somata of acutely dissociated TRG neurons

In order to determine whether ATP can be released directly from somata of TRG neurons, we exposed the neuron-rich glass tubes to capsaicin (5 μM). This evoked a robust increase in ATP release (Fig. 5). Exposure to MEM vehicle without capsaicin did not change ATP levels. Observation of the neuron-rich sister cell suspensions plated on coverslips revealed that none of the neurons had any visible processes emanating from the somata. Counts from these coverslips showed that the proportion of neurons to non-neuronal cells was 69:14 (83% neuron content). These data suggested that ATP is principally released directly from the somata of sensory neurons.

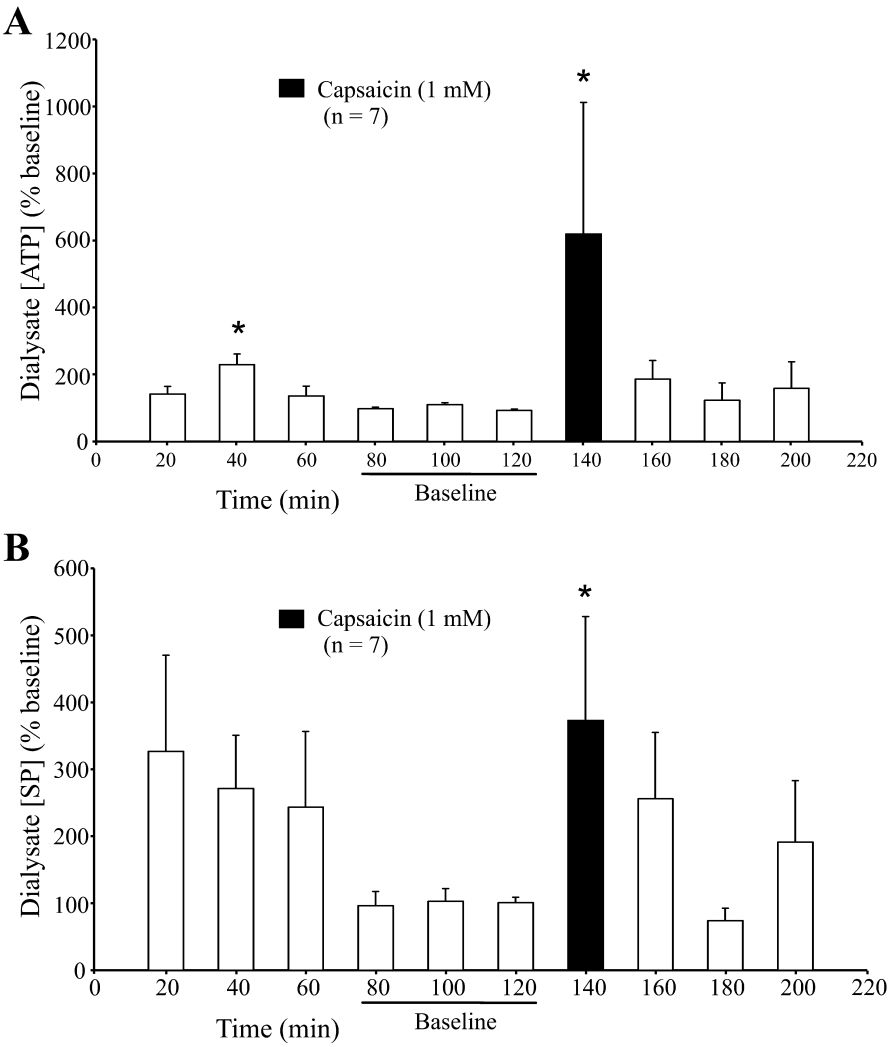

Fig. 2. Concurrent release of ATP and substance P within TRG evoked by selective neuronal activation with capsaicin. (A) Summary of seven experiments measuring ATP content before and after infusion of 1 mM capsaicin. Actual baseline [ATP] was 108.2±18.7 pM. (B) Summary graph of SP levels measured in the same sample after ATP analysis. Actual baseline [SP] was 47.6±20.1 pM. Asterisks denote significant difference ($P<0.05$) from the last sample before capsaicin infusion. Baseline was measured as the average ATP or SP in the three samples starting 1 h prior to capsaicin infusion.

4. Discussion

The major findings of this study are: (1) 100 mM KCl or 1 mM capsaicin evoke concurrent release of ATP and SP within the TRG, (2) Ca^{2+}-free, EGTA-containing ACSF produces large increases in ATP levels, (3) KCl-induced release of ATP is blocked by Cd^{2+}, a voltage-gated Ca^{2+} channel blocker, and (4) capsaicin evokes ATP release from acutely dissociated somata of TRG neurons.

This is the first report to demonstrate concurrent release of ATP and SP within sensory ganglia in vivo. Both ATP and SP release was evoked with capsaicin, a selective activator of C- or Aδ-fiber sensory neurons [16]. Capsaicin also evoked ATP release from the somata of neuron-rich dissociated cells in this study and SP release from the somata of acutely dissociated DRG neurons has been demonstrated previously [17]. Another recent study demonstrated that the evoked release of calcitonin gene-related peptide (CGRP) from slices of trigeminal nerve is very weak compared to release from slices of the TRG [38]. Thus, in vivo release of ATP and SP in our study most likely occurred directly from the somata of neurons in within the TRG.

The precise sequence of ATP and SP release remains to be determined. In some systems SP was shown to induce ATP release [36], while in others ATP release may precede SP release [28]. Furthermore, the existence of two major subpopulations of nociceptive sensory neurons with distinct peripheral and central innervation patterns, neuropeptide content, and lectin binding characteristics is now well established [13,22,23,30,40], yet the somata of both neuronal subpopulations possess capsaicin receptors [13]. Thus, our data do not in any way preclude the possibility that the capsaicin-evoked release of ATP and SP occurs separately from distinct populations of sensory neurons. Alternatively, since it has been reported that ATP is

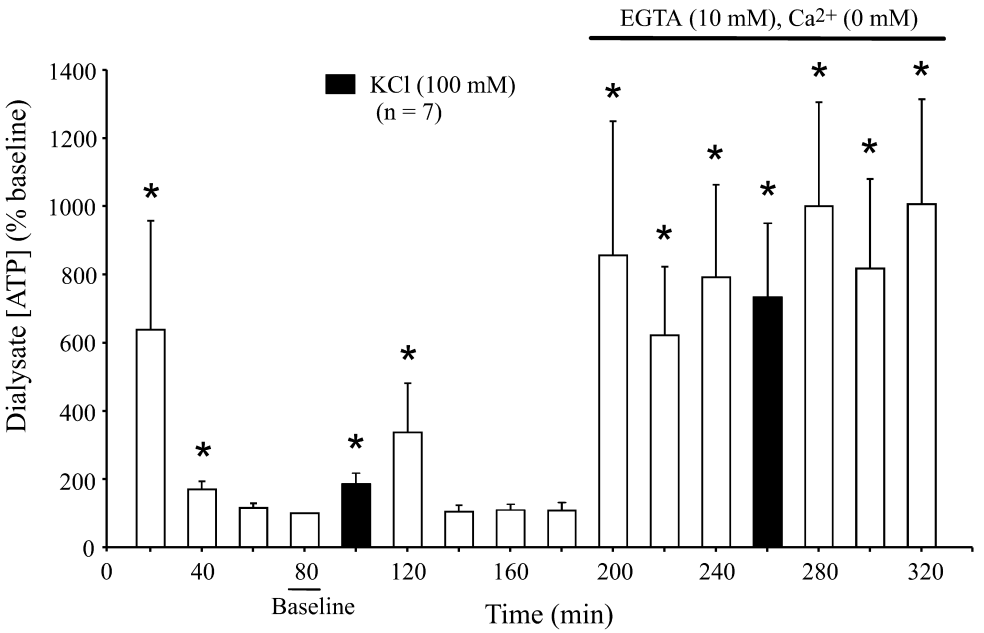

Fig. 3. Effects of Ca^{2+}-free, EGTA perfusate on ATP release within TRG. Summary graphs of ATP content in microdialysis samples from TRG as determined by the L/L assay. The probes were perfused with normal ACSF followed at 80 min by a 20 min perfusion with 100 mM KCl-containing ACSF. They were switched to $CaCl_2$-free, EGTA-containing ACSF at 180 min. At 240 min the perfusate was switched for 20 min to 100 mM KCl and $CaCl_2$-free, EGTA-containing ACSF. Baseline was taken as the last sample prior to KCl infusion. Bars represent mean % of baseline±S.E.M. Actual baseline [ATP] was 135.9±80.3 pM. Asterisks denote significant increase ($P<0.05$) from the baseline by repeated measure ANOVA.

co-released with other neurotransmitters [18,31,34], ATP and SP may be co-released from the same neurons and possibly from the same vesicles. We recently reported that SP release within TRGs is greatly increased 48 h after unilateral orofacial inflammation [24]. It would be of interest to determine whether extracellular ATP levels are similarly increased after inflammation.

Large increases in ATP content were observed after perfusion with Ca^{2+}-free, EGTA-containing ACSF. It has been reported that ATP is hydrolyzed to adenosine by ecto-nucleotidases, which require Ca^{2+} and Mg^{2+} for activation [39,42]. Certain ecto-nucleotidases have a greater dependency on Ca^{2+} than Mg^{2+} [27]. A decrease in extracellular [Ca^{2+}] should result in decreased ATP hydrolysis, thereby resulting in the increased ATP levels seen with EGTA and Ca^{2+}-free ACSF. Since we maintained

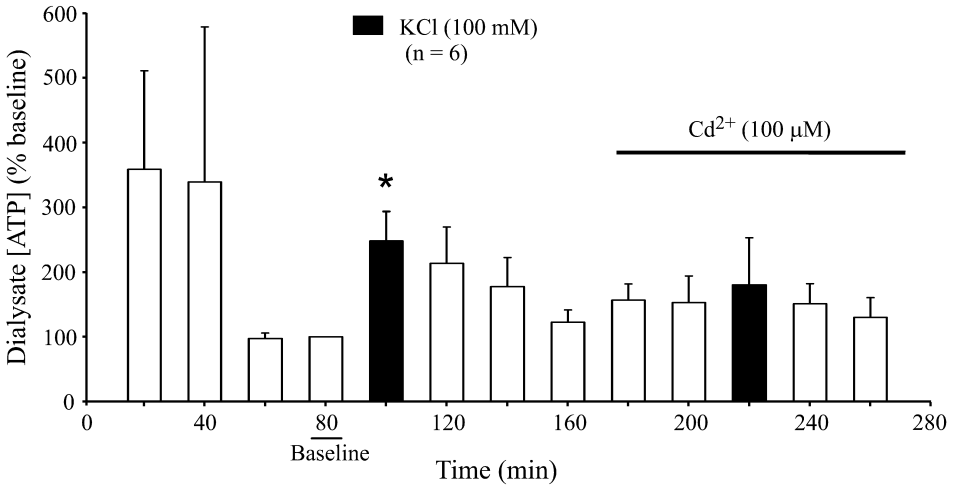

Fig. 4. Calcium dependence of KCl-evoked ATP release in trigeminal ganglia. Summary graph of ATP content in microdialysis samples from trigeminal ganglia. The probes were perfused with normal ACSF followed at 80 min by a 20 min perfusion with 100 mM KCl-containing ACSF. They were switched to ACSF with 100 μM $CdCl_2$ at 160 min. At 200 min the perfusate was switched for 20 min to 100 mM KCl and 100 μM $CdCl_2$-containing ACSF. Baseline was taken as the last sample prior to KCl infusion. Actual baseline [ATP] was 102.3±14.6 pM. Asterisks denote significant increase ($P<0.05$) from the baseline. 100 mM KCl-containing ACSF did not induce significant increase of SP release in 100 μM $CdCl_2$-containing ACSF condition.

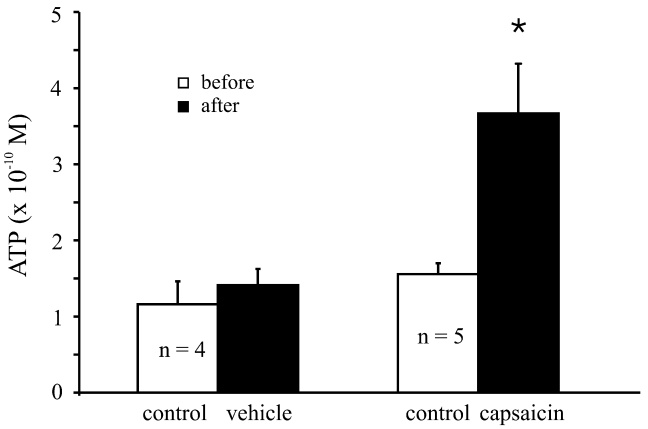

Fig. 5. Capsaicin evokes ATP release from somata of acutely isolated sensory neurons. ATP concentration was measured using the L/L assay before (open bars) and after (solid bars) addition of capsaicin (5 μM) or vehicle to the test tubes containing plated neurons. Asterisk denotes significant difference ($P=0.02$) in ATP levels.

divalent cation and chloride ion balance with $MgCl_2$ in the Ca^{2+}-free ACSF, Ca^{2+}-dependent ecto-ATPase may be important in metabolism of extracellular ATP within the TRG. To avoid interference with ecto-nucleotidases, we used Cd^{2+} to block the Ca^{2+} influx through voltage-gated Ca^{2+} channels after high K^+ stimulation [14]. Data from this experiment corroborated the ecto-nucleotidase explanation, since application of Cd^{2+} without changing extracellular [Ca^{2+}] and [Mg^{2+}] did not increase basal ATP levels. However, Cd^{2+} application did block the high K^+-induced ATP release, confirming its dependence on voltage-gated Ca^{2+} channels. Interestingly, Cd^{2+} application did not affect basal ATP release (Fig. 4), in contrast to the previously reported decreases in the basal release of SP [24] or CGRP [38] during application of low Ca^{2+}/EGTA containing solutions. This suggests that the basal release of ATP from sensory neurons is independent of Ca^{2+} influx through voltage-dependent Ca^{2+} channels, and therefore may be governed by mechanisms different from those mediating basal release of neuropeptides.

The hypothesis that cross-depolarization in sensory ganglia is chemically mediated implies that a substance is released by the activity of one neuron, diffuses to another neuron and influences its activity via activation of receptors on its plasma membrane [1]. In this study we provided evidence for the ganglionic release of ATP and SP, thereby fulfilling one of the criteria for a putative chemical mediator of cross-excitation. However, the identity and function of receptors for ATP and SP on the soma of sensory neurons are a matter of considerable controversy. For example, P2X receptors have been immunocytochemically detected on the somata of neurons in sensory ganglia [25]. However, while the vast majority of acutely dissociated DRG neurons respond to exogenous ATP, application of ATP in whole-mount DRG preparations has little effect on DRG neurons [35]. In contrast, SP does evoke electrophysiological responses in neurons in whole-mount DRG preparations as well as in sensory neurons in culture [9,29], yet receptor binding and immunocytochemistry studies have failed to demonstrate the presence of receptors for substance P in sensory ganglia [4,21]. Thus, to establish ATP or SP as mediators of cross-excitation, it is clear that future experiments would need to provide a direct causal link between activation of ATP or SP receptors and cross-excitation of neurons within sensory ganglia.

In summary, the present study provides evidence for concurrent release of ATP and substance P from sensory neurons within the TRG in vivo. Given the ability of ATP and substance P to depolarize the somata of sensory neurons, we propose that both ATP and substance P could serve as chemical messengers that may mediate non-synaptic cross-excitation between neurons in sensory ganglia.

Acknowledgements

We thank Dr. Christopher J. Evans for the generous gift of the substance P antibody. We thank Soon H. Lee and Aaron Fang for help with the ATP measurements. Special thanks to Drs. Lawrence Kruger and Felix Schweizer for helpful advice, and Dr. Schweizer for the use of his tissue culture facilities. This work was supported by NIH grants DE07212, DE00408, and the Whitehall Foundation.

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